JPP
Journal of Pharmacy
And Pharmacology

Review

# Insights into the effects of diclofenac and other non-steroidal anti-inflammatory agents on ion channels

Asfree Gwanyanya[a], Regina Macianskiene[b] and Kanigula Mubagwa[c]

[a]Department of Human Biology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, [b]Laboratory of Membrane Biophysics, Institute of Cardiology, Kaunas University of Medicine, Kaunas, Lithuania, and [c]Department of Cardiovascular Diseases, University of Leuven, Leuven, Belgium

**Keywords**
diclofenac; ion channel; non-steroidal anti-inflammatory drug

**Correspondence**
Kanigula Mubagwa, Campus Gasthuisberg, Herestraat 49, Box 705, B-3000, Leuven, Belgium.
E-mail: kanigula.mubagwa@med.kuleuven.be

Received July 13, 2011
Accepted January 5, 2012

doi: 10.1111/j.2042-7158.2012.01479.x

## Abstract

**Objectives** Diclofenac and other non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of inflammation and pain. Most effects of NSAIDs are attributed to the inhibition of cyclooxygenases (COX). However, many NSAIDs may have other effects not related to COX, including the modulation of various ion channels. The clinical implications of the effects on channels are not fully understood. This review outlines the effects of NSAIDs, with special attention to diclofenac, on ion channels and highlights the possible underlying mechanisms.

**Key findings** NSAIDs have effects on channels such as inhibition, activation or changes in expression patterns. The channels affected include voltage-gated $Na^+$, $Ca^{2+}$, or $K^+$ channels, ligand-gated $K^+$ channels, transient receptor potential and other cation channels as well as chloride channels in several types of cells. The mechanisms of drug actions not related to COX inhibition may involve drug–channel interactions, interference with the generation of second messengers, changes in channel expression, or synergistic/antagonist interactions with other channel modulators.

**Summary** The effects on ion channels may account for novel therapeutic actions of NSAIDs or for adverse effects. Among the NSAIDs, diclofenac may serve as a template for developing new channel modulators and as a tool for investigating the actions of other drugs.

## Introduction

### Anti-inflammatory action and cyclooxygenase inhibition

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed agents for analgesic, antipyretic and anti-inflammatory purposes, with many of the drugs being readily available over-the-counter. They include such various substances as salicylates (aspirin), aryl propionic derivatives like ibuprofen, aryl acetic derivatives like diclofenac, indole derivatives like indometacin, oxicams like piroxicam, melusid, phenylbutazone, etc. As initially proposed by Vane[1], these structurally diverse chemicals have in common the fact that they inhibit cyclooxygenase (COX) enzymes, which catalyse the conversion of arachidonic acid to prostaglandin (PG) $G_2$ and eventually to $PGH_2$, a precursor of thromboxane $A_2$ and other effector prostaglandins (for reviews see Botting[2] and Vane *et al.*[3]). At least two distinct isoforms of COX are known to exist: the constitutive COX-1 found in platelets, kidneys, gut and other tissues, and the inducible COX-2 produced locally in response to inflammatory signals, growth factors, cytokines, oncogenes and other stress signals.[4,5] The existence of different COX isoforms has stimulated the development of new isoform-selective NSAIDs. However, most of the drugs commonly used in the therapy are COX nonselective (i.e. they inhibit both COX-1 and COX-2).

### Other actions of NSAIDs: COX and other effectors

Besides their use in pathological processes related to inflammation, NSAIDs have been proposed for use in treating other disease processes. For example, low-dose aspirin is widely used for its anti-platelet aggregation effect, due to its inhibition of COX.[6] NSAIDs have also been suggested to exert beneficial effects in cancer and in neurodegenerative disorders such as Alzheimer's disease.[7–9] The anticarcinogenic effect may involve an inhibition of tumour growth, a reduction of

metastases, or an improved response to treatment by other antitumour agents. The mechanisms underlying these antitumour effects are complex and largely unknown. Some, but not all anticancer effects may be related to the expression of COX-2 in cancer cells and include such various processes as the modification of the expression level of proteins associated with apoptosis or the suppression of an upregulation of the multidrug resistance-associated proteins.[10,11]

With the increased use of the nonselective NSAIDs like indometacin, naproxen and ibuprofen, life-threatening adverse effects such as gastrointestinal mucosal erosion/perforation, renal impairment and platelet dysfunction also became common.[12,13] These adverse effects, particularly gastrointestinal toxicity, have been attributed to the loss of physiological PGs due to inhibition of COX-1 and not COX-2, and clinically COX-2-selective drugs such as rofecoxib and lumiracoxib show substantially lower incidences of gastric ulceration compared with naproxen or ibuprofen.[14–17] It is therefore understandable that there had been a general trend towards marketing COX-2-selective NSAIDs. However, these drugs also have adverse effects.

A promising COX-2 selective NSAID rofecoxib was shown to be associated with an increased risk of myocardial infarction and cerebrovascular thrombo-embolic accidents in a multicentre trial, a finding that was supported by other studies and which led to the withdrawal of the drug from the market in 2004.[18–20] The following year, a related drug, valdecoxib, was also withdrawn. The COX-2-selective drug celecoxib remains in use, but it has been reported to be associated with cardiovascular risk.[21,22] The basis of these adverse effects is unclear, but COX-2-selective drugs have been proposed to promote atherogenesis by interfering with the metabolism of low density lipoproteins and of vascular cell membrane phospholipids, or by creating an imbalance between endothelial COX-2-dependent prostacyclin ($PGI_2$) and platelet COX-1-dependent thromboxane $A_2$ (see Joshi *et al*.[23] and Capone *et al*.[24]). There is now therefore less drive towards COX-2-selective drugs, making it likely that nonselective NSAIDs with relatively less gastrointestinal toxicity may retain clinical prominence.[25,26] However, the increased risk for cardio- and cerebrovascular events is not specific for COX-2-selective inhibitors but also concerns nonselective NSAIDs.

Besides COX inhibition, the mechanisms underlying various potential therapeutic or adverse effects of NSAIDs remain unclear. Several NSAIDs have been shown to modulate ion channels, and there is a possibility that these effects on ion channels could account for the drug actions not directly linked to COX inhibition. Among the effects of NSAIDs on ion channels, those of diclofenac have been the most extensively studied. Diclofenac (2-[2-(2,6-dichlorophenyl)aminophenyl]acetic acid) has structural components similar to those found in other NSAIDs and in functionally different compounds such as diphenhydramine or carbamazepine (see Figure 1a for chemical structures). Diclofenac has been in clinical use as an anti-inflammatory agent for over three decades, and has been proposed to have other beneficial effects like central analgesia and anticancer activity.[27–29] Besides the typical NSAID-related adverse effects, diclofenac is also implicated in hepatic and renal toxicity as well as in increased cardiovascular risk.[19,20,30–32] This article outlines the effects of diclofenac and other NSAIDs on ion channels and highlights the possible mechanisms underlying the drug actions. Some new experimental data from studies on cardiac myocytes have been presented.

## Effects of diclofenac and other NSAIDs on ion channels

As mentioned above, many members of the NSAID group are reported to exert effects on ion channels in various native tissues or cells and in expression systems. Some NSAIDs are even frequently used as pharmacological tools to identify ion currents due to particular classes of channels (e.g. fenamates, used to identify anion channels). Most of these effects on ion channels occur at concentrations much higher than those known to inhibit COX, and so it can be assumed that the actions on channels are nonspecific and of little relevance to clinical conditions. However, in-vivo concentrations of some NSAIDs may reach millimolar levels, making ion channel effects potential contributors to therapeutic effects. In addition, based on the demonstrated beneficial effects of NSAIDs in animal models in which COX has been suppressed, or on the evidence for pharmacological effects of NSAID-related drugs not known to have action on COX, as well as on the heterogeneity of effects among NSAIDs, there is increasing awareness of the possible role of non-COX targets (such as ion channels) as potential players in the beneficial effects of NSAIDs.

The effect of NSAIDs on ion channels are not selective, since practically all those found to act on channels affect many channel types. Among NSAIDs, salicylic acid has been reported to modulate such diverse channels as voltage-dependent $Na^+$, $K^+$ or $Ca^{2+}$ channels (described below), as well as channels associated with the $GABA_A$, glycine and NMDA 3 receptors.[33–35] Recent studies indicated that the drug inhibited acid-sensing ion channels.[36] Fenamates, which include the clinically used niflumic acid, are known to inhibit various $Cl^-$-selective channels, but they are also used to inhibit many cation-nonselective channels. In addition, they may show inhibitory effects on HCN channels or stimulatory effects on 4 other channels, including $K^+$ channels.[37,38]

The basis for this pleiotropic action on channels remains unclear. One mechanism could implicate an indirect action by interfering with COX, resulting in the elimination of the effects of COX products or in the enhancement of the effects

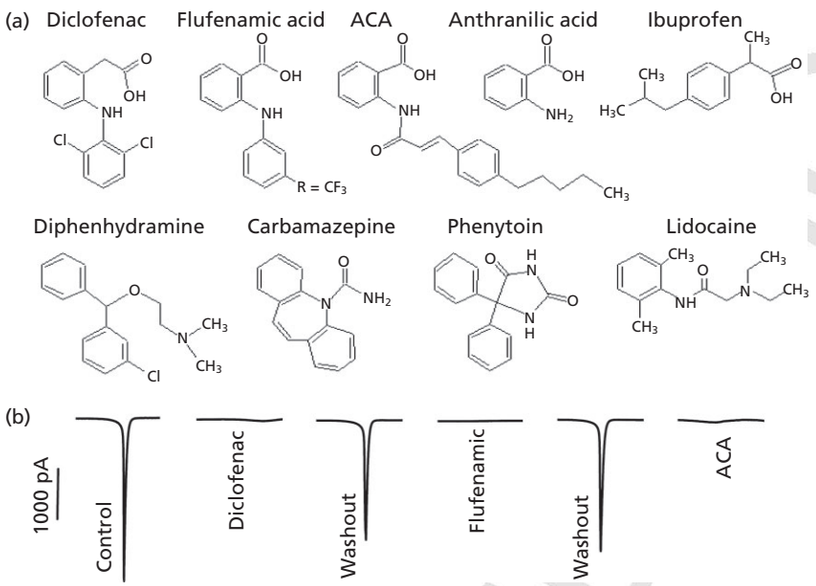



**Figure 1** Diclofenac and related drugs: suppression of the voltage-gated $Na^+$ current in cardiac myocytes. (a) Chemical structures of diclofenac and other drugs. Notice (1) the anthranilic acid moiety in its derivatives *N*-(p-amylcinnamoyl)anthranilic acid (ACA) and flufenamic acid, but a modified structure in diclofenac. (2) Diclofenac, ibuprofen, and anthranilic acid and anthranilic acid derivatives contain a carboxylate (COOH) group. (3) Diclofenac, flufenamic acid, diphenhydramine, carbamazepine and phenytoin contain a diphenyl group. (b) Original traces of $Na^+$ currents ($I_{Na}$) from patch clamp recordings in a pig ventricular myocyte. Currents were obtained using ramps in a cell dialysed and superfused with $K^+$-free solutions containing physiological levels of $Na^+$. Diclofenac (100 μM), flufenamic acid (100 μM), and ACA (20 μM) were applied extracellularly and separately.

of endogenous arachidonic acid (Figure 2). However, as discussed below, many effects on channels are due to a direct action on the channel proteins. These direct effects may involve a modification of channel gating properties, an effect on pore permeation, or a change in channel protein expression (Figure 2). Nonetheless, many channels are insensitive to NSAIDs, whereas the effects on others are still yet to be evaluated.

The channels affected by NSAIDs include voltage-gated $Na^+$, voltage-gated L-type $Ca^{2+}$, voltage- and ligand-gated $K^+$, transient receptor potential (TRP), and others (see Table 1 for a summary of diclofenac effects).

## Voltage-gated $Na^+$ channels

Voltage-gated $Na^+$ ($Na_V$) channels underlie the initial depolarization phase of the action potential in many excitable cells, and modulation of these channels constitutes the basis for the treatment of pain and of hyperexcitability conditions such as epilepsy or cardiac arrhythmias. As mentioned above, part of these therapeutic actions may be due to COX inhibition. For example, flufenamic acid and indometacin suppress the increase of $I_{Na}$ caused by intracellular PGs produced following cell dialysis with arachidonic acid.[61] But other effects involve a direct interaction with the channel protein.

Indometacin, aspirin or antipyrin did not change $I_{Na}$ in dorsal root ganglion (DRG) neurons nor did nepafenac and amfenac change the current in trigeminal ganglion neurons.[39,42] Aspirin also has no effect on $Na_V$ channels in mice spinal ganglion neurons.[35] However, in rat pyramidal neurons, the aspirin metabolite salicylate inhibited $I_{Na}$ by shifting the steady-state inactivation to more negative potentials.[62] Ibuprofen or the COX-2 selective inhibitor NS 398 decreased the inflammation-induced upregulation of $Na_V$ 1.7 channels in DRG neurons.[63] This effect was indirect since it was related to the suppression of PG formation. Another COX-2 selective inhibitor, celecoxib, also decreased the expression of tetrodotoxin (TTX)-resistant $Na_V$ channels (i.e. channels displaying low sensitivity to TTX) in similar types of cells.[64]

Electrophysiological effects of diclofenac have been most extensively characterized on voltage-gated $Na^+$ ($Na_V$) channels. The drug decreases the peak amplitude of currents carried by several types of $Na_V$ channels in various cells, without changing activation kinetics or ion selectivity (as indicated by the lack of change of the reversal potential). The affected $Na_V$ channels include TTX-sensitive channels in DRG neurons and in myoblasts as well as TTX-resistant channels in DRG neurons.[39,40] The drug also blocks $Na^+$ channels in brain CA1 region neurons and in trigeminal

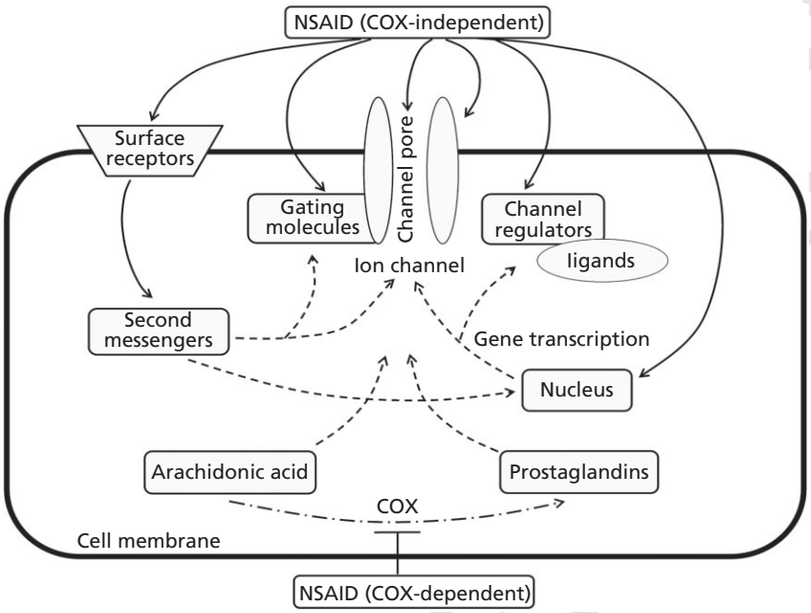



**Figure 2** Cellular actions of non-steroidal anti-inflammatory drugs on ion channels. Cyclooxygenase (COX)-dependent and COX-independent pathways of actions of non-steroidal anti-inflammatory drugs (NSAIDs) on cell membrane ion channels. The cascades of interactions are indicated by bold and dotted lines. NSAIDs act on ion channels directly or indirectly, via intracellular molecules. Cross-talk between the two pathways may also occur.

ganglion neurons.[41,42,65] The effect of diclofenac on $Na^+$ channels in nervous tissue is reversible. In DRG neurons, the potency to block TTX-sensitive $Na^+$ currents ($I_{Na}$) (7) ($K_d = 14\ \mu M$) appeared higher than for TTX-resistant $I_{Na}$ ($K_d = 97\ \mu M$).[39]

The effect on peak current amplitude in brain CA1 region neurons depends on the holding membrane potential before depolarization: it is absent if the holding potential is very negative, but it increases with more depolarized holding potentials.[41,65] This indicates that the effect of diclofenac is channel state dependent. The lack of an effect with very negative holding potentials suggests that the drug has little affinity for the resting state, whereas the decrease of peak current with less negative holding potentials and the use-dependent suppression of $I_{Na}$ indicate an interaction with the inactivated and/or the activated channel states.[41,65] The binding to the inactivated state of the channel is reflected by a shift of the inactivation curve to hyperpolarized potentials.[39,41] The affinity for the inactivated state of the channel ($K_{inactive} = 7\ \mu M$), obtained from the magnitude of the shifts in steady-state voltage-dependent inactivation (or availability) curves, is at least two-orders of magnitude higher compared with the affinity for the resting state ($K_{resting} = 880$–$1500\ \mu M$).[41] An interaction with the open state is suggested by the slowing of $Na^+$ current decay in the presence of diclofenac. It can be further demonstrated by a voltage-dependent increase in current amplitude when inactivation is suppressed in $Na^+$ channel mutants expressed in heterologous systems. In these inactivation-deficient mutants, the inhibitory effect of diclofenac is lost, and instead the drug actually enhances $I_{Na}$, suggesting a stabilization of the open state of the channel.[41] The drug has been proposed to induce allosteric changes via a binding site in the ion conducting pathway accessible from outside the cell, since the blocking effects are modulated by extracellular, but not by internal $Na^+$.[41]

The above effects of diclofenac on $Na^+$ channels are not surprising given that it contains a diphenyl structure similar to the one present in other drugs, such as carbamazepine, phenytoin and lamotrigine, which also act on $Na^+$ channels. Diclofenac and carbamazepine seem to share a common binding site, since their effects are mutually exclusive.[41] However, there are also differences between these two drugs: whereas both drugs also bind to the open state, carbamazepine causes an open state channel block in the presence of extracellular $Na^+$ (resulting in an accelerated decay of the macroscopic $Na^+$ current), in contrast to diclofenac, which, as discussed above, stabilizes the open state and causes a slight slowing of macroscopic current decay.[41]

Due to the failure of some NSAIDs to inhibit $I_{Na}$, the suppressive effects of diclofenac in neurons appear to take place independently of COX inhibition. This finding suggests that although COX inhibition is an essential component of NSAID effect, COX-independent effects such as the

**Table 1** Diclofenac effects on ion channels

| Ion channel | | Tissue/cell type | Diclofenac effect | $K_d$, IC50 or EC50 | Key references |
|---|---|---|---|---|---|
| $Na^+$ channels | Voltage-gated $Na^+$ channels | Dorsal root ganglion (DRG) neurons (TTX-sensitive) | Suppression of $Na^+$ currents | 14 μM ($n_{Hill} \approx 0.8$) | [39] |
| | | DRG neurons (TTX-resistant) | | 97 μM ($n_{Hill} \approx 1.57$) | [39] |
| | | Myoblasts (TTX-sensitive) | | 8.5 μM ($n_{Hill} \approx 0.36$) | [40] |
| | | CA1 region neurons | | 7 μM ($K_{inactivation}$) | [41] |
| | | Trigeminal ganglion neurons | | | [42] |
| $Ca^{2+}$ channels | L-type $Ca^{2+}$ channels | Neonatal ventricular myocytes | Suppression of $Ba^{2+}$-mediated currents | ≈ 12.9 μM | [43] |
| | | Vascular smooth muscle cells | No effect | | [44] |
| $K^+$ channels | | | | | |
| Voltage-gated | $K_V1.2$, $K_V1.5$ or $K_V2.1$ | Chinese hamster ovary (CHO) cells or macrophages | None | | [45,46] |
| | $K_V1.3$ | Macrophages or T-lymphocytes | Suppression of baseline $K_V1.3$ currents in lipopolysaccharide- activated macrophages<br>Suppression of $K_V1.3$ mRNA and protein expression in activated macrophages and T-lymphocytes | 2.6 μM ($n_{Hill} \approx 1.3$) | [45] |
| | Transient outward $K^+$ channels ($I_A$) ?$K_V4.x$ | Cerebellar granule neurons | Activation of $I_A$ | | [47] |
| | | CHO cells | Activation of M-current | | |
| | Muscarinic receptor (M-type or $K_V7.2/3$) $K^+$ channels | Cortical neurons | Suppression of spontaneous or evoked neuronal activity | | [46] |
| | $K_V7.4$ and $K_V7.5$ | Vascular smooth muscle cells | Suppression of $K_V7.5$, but enhancement of $K_V7.4$ currents | | [48] |
| Ligand-gated | ATP-sensitive $K^+$ ($K_{ATP}$) channels,<br>$Ca^{2+}$-activated $K^+$ ($K_{(Ca)}$) channels | Systemic effects | ? Nitric oxide-cGMP-mediated activation of $K^+$ channels (decreased pain responses) | | [49–54] |
| | $Ca^{2+}$-regulated $K^+$ (? EAG) channels | Melanoma cells | Activation of channels | | [55] |
| Acid-sensing ion channels (ASIC) | Native ASIC channels and expressed ASIC1a/2a, ASIC2a or ASIC3 channels | Hippocampal interneurons, DRG neurons, bone marrow-derived dendritic cells, COS cells or CHO cells | Suppression of ASIC currents<br>Reduction of the expression of inflammation-induced ASIC mRNA in DRG neurons | 92 μM for COS cells<br>622 μM for interneurons | [36]<br>[56]<br>[57]<br>[36] |
| Transient receptor potential (TRP) and other cation nonselective channels | TRPA1 channels | Human lung fibroblasts | Increase in TRPA1-mediated $Ca^{2+}$ loading | 210 μM ($n_{Hill} \approx 1.3$) | [58] |
| | Fenamate-sensitive channels | *Xenopus* oocytes | Activation of TRPA1 channels | | [59] |
| | Stress-activated, $Ca^{2+}$-permeable channels | Cardiac myocytes | None | | [60] |
| | | Erythrocytes | Suppression of the cation current induced by either prostaglandin $E_2$ or the removal of extracellular chloride | | |

$K_d$, dissociation constant; IC50 or EC50, concentration at 50% inhibition or stimulation, respectively; $n_{Hill}$, Hill coefficient; TTX, tetrodotoxin; ?, depicts uncertainty or lack of data.

inhibition of $I_{Na}$ could contribute to the known antinociceptive effects of some NSAIDs.[66,67]

In myoblasts, diclofenac also shows a greater block of TTX-sensitive $I_{Na}$ at less negative potentials and shifts the inactivation curve to more negative potentials.[40] Unlike in the above-mentioned CA1 region neurons, diclofenac also inhibits $I_{Na}$ in myoblasts when applied from the intracellular compartment.[40] Furthermore, the blocking effects of the drug applied from inside and outside the cell are additive, suggesting the presence of distinct drug binding sites. Here also, the effect of extracellular diclofenac exhibits a frequency-dependence as in neurons. To account for the frequency-dependency of the block, the authors proposed that there was a fast binding of the drug to the channel, followed by a slow unbinding process, as is described for local anaesthetics (see Butterworth *et al*.[68]).[40] However, other factors may play a role given the presence of other complex effects such as the spontaneous partial recovery from the block observed in the same cells.[40]

The effects of diclofenac and of other NSAIDs in cardiac cells have so far received little attention despite the potential cardiovascular side effects of the drugs. A reversible block of $I_{Na}$ by diclofenac, but not by other COX inhibitors naproxen and ibuprofen, has been reported in neonatal ventricular myocytes.[43] However, given that the afore-mentioned study was meant to investigate the effects of diclofenac on $Ca^{2+}$ channels (see section on $Ca^{2+}$ channels below), not much detail is known about this action on $I_{Na}$. We therefore used whole-cell patch-clamp measurements in isolated adult ventricular myocytes (as described in Gwanyanya *et al*.[69]) to further evaluate the effects of diclofenac on $I_{Na}$. Figure 1b shows that diclofenac (100 μM) suppressed $I_{Na}$ activated during a depolarizing voltage ramp protocol. Similar effects can be obtained with the structurally related, but functionally different drugs flufenamic acid (also a COX inhibitor) and *N*-(p-amylcinnamoyl)anthranilic acid (ACA, a phospholipase $A_2$ inhibitor) (Figure 1b): like diclofenac, flufenamic acid (100 μM) or ACA (20 μM) each inhibited $I_{Na}$ and the effects were reversible upon drug washout. Figure 3a illustrates the diclofenac effect on $I_{Na}$ induced by a depolarizing voltage step protocol, whereas Figure 3b shows that the drug decreased the action potential duration and the rate of maximal depolarization during the upstroke (Figure 3b, lower panel), without affecting the resting potential. We also observed a decrease of the action potential overshoot amplitude (not illustrated; but see Macianskiene *et al*.[59]). These effects are all consistent with a decrease of $I_{Na}$. Previously, we reported that ACA, like diclofenac, reduced peak amplitudes of ventricular action potentials, an effect consistent with the block of $I_{Na}$ reported here.[59] The above results indicated that, like neuronal $Na_V$ channels, cardiac $Na_V$ channels were also sensitive to a direct action of diclofenac and of fenamate NSAIDs.

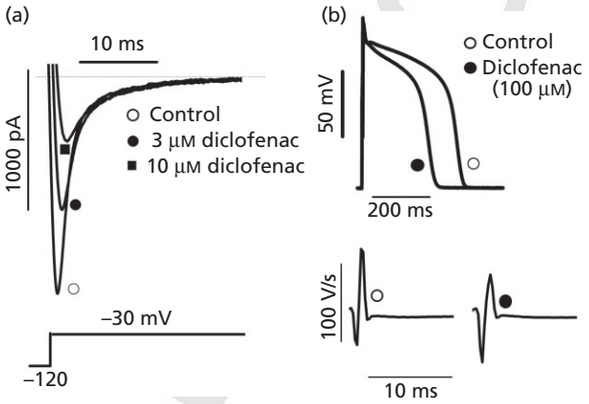



**Figure 3** Suppression of the voltage-gated $Na^+$ current and effects on action potential and on the rate of initial depolarization in cardiac myocytes. (a) Traces of voltage-dependent $Na^+$ currents ($I_{Na}$) induced at –30 mV from a holding potential of –120 mV, in control conditions and in the presence of increasing concentration of diclofenac. Cell dialysed with physiological internal solution and superfused with $Na^+$-containing solution. The lower panel shows the voltage step protocol. Notice suppression of $I_{Na}$ by diclofenac. (b) Action potentials (upper panel) and their first time derivatives (lower panels), recorded before (open circle) and during application (solid circle) of diclofenac (100 μM). Ruptured-patch, whole-cell recording. $K^+$ used in both internal and external solutions. Notice the decrease of action potential duration and the slowing of the rate of upstroke.

## Voltage-gated $Ca^{2+}$ channels

Currently, not much is known about the effects of NSAIDs, including diclofenac, on voltage-dependent $Ca^{2+}$ channels. Salicylate inhibited $I_{Ca}$ in rat pyramidal or inferior colliculus neurons.[62,70] In those cells, the drug shifted the steady-state inactivation curve to the left and delayed the recovery from inactivation.

In a study involving rat neonatal ventricular myocytes, diclofenac has been reported to suppress whole-cell L-type $Ca^{2+}$ currents ($I_{Ca\text{-}L}$) for which $Ba^{2+}$ was the charge carrier.[43] Those authors proposed that the reported diclofenac effects were independent of COX inhibition, based on the lack of an effect of naproxen or ibuprofen on $I_{Ca\text{-}L}$ or on $I_{Na}$ observed in their preliminary experiments. The decrease of the $Ba^{2+}$-mediated $I_{Ca\text{-}L}$ obtained in the presence of diclofenac was irreversible, but the reason for this irreversibility in the presence of a reversible effect on $I_{Na}$ remains unclear. It cannot be excluded that the observed decrease of $I_{Ca\text{-}L}$ was simply due to run-down of the current and not to a pharmacological effect, since no clear control of the kinetics of run-down with or without diclofenac was mentioned in the study. Nonetheless, in contrast to the effects in neonatal cardiac myocytes, diclofenac has no effect on depolarization-induced $Ca^{2+}$ influx or on $I_{Ca\text{-}L}$ in vascular smooth muscle cells, probably depicting tissue-specific differences in the

drug sensitivity.[44,71] Clearly, more studies are needed to clarify the issue of the effect of diclofenac on $I_{Ca\text{-}L}$ in cardiomyocytes and the potential contribution of an eventual effect to the observed shortening of action potential.

## $K^+$ channels

### *Voltage-gated $K^+$ channels*

NSAIDs modulate a variety of natively- or heterologously-expressed voltage-gated $K^+$ channels. For diclofenac, the sensitive channels include $K_V1.3$ channels, transient outward ($I_A$ or $K_V4.x$) $K^+$ channels and muscarinic receptor-inhibited (M-type or $K_V7.2/3$) $K^+$ channels.[45–47,55,72] However, other voltage-gated $K^+$ channels such as $K_V1.2$, $K_V1.5$, and $K_V2.1$ channels are unaffected by diclofenac.[45,46]

### *$K_V1.x$ and $K_V2.x$ currents*

The NSAIDs indometacin, phenylbutazone and NS 398, but not the COX-1 selective inhibitor SC 560, decrease the surface expression of heteromeric $K_V1.4$ and $K_V1.6$ channels in intestinal epithelial cells.[73] Celecoxib, but not rofecoxib, inhibits $K_V1.5$ channels (as well as $K_V4.3$ and $K_V7.1$ channels) expressed in mammalian cells and alters action potential durations of ventricular myocytes.[74] Celecoxib also inhibits heterologously-expressed $K_V2.x$ channels and induces arrhythmias in rat ventricular myocytes.[75] In contrast, acetylsalicylic acid (aspirin) does not have similar effects in the same type of cells. However, salicylate inhibits the delayed rectifier current in rat pyramidal cortical neurons by shifting the steady-state inactivation to more negative potentials.[62]

Diclofenac has been reported to suppress both $K_V1.3$ currents and channel protein expression as well as to alter the cell migration patterns in leucocytes.[45] In that study, diclofenac inhibited whole-cell $K_V1.3$ currents in activated macrophages, decreased the interleukin-2 production in activated T-lymphocytes, and downregulated the $K_V1.3$ mRNA and protein expression. Functionally, the drug impairs the migration of activated macrophages, an effect of diclofenac previously reported for other leucocytes.[45,76–78] The mechanisms underlying the $K_V1.3$ channel-mediated drug actions remain unclear, but may involve the downregulation of stress signal sensors like the inducible nitric oxide synthase (iNOS).[45] Those authors have proposed that anti-inflammatory actions of the drug may include a component due to immune modulation.

### *$K_V4.x$ currents*

A fast transient outward current is present in neuronal cells and is carried by $K_V4$ channels. Similar transient outward currents are present in other tissues, including cardiac cells, where the underlying channel types and expression levels are species-dependent but may involve $K_V4.x$ channels (see Amuzescu *et al*.[79]).

Little is known about the effect of most NSAIDs on $K_V4$ channels. Diclofenac increases the amplitude of the fast transient outward ($I_A$ or $K_V4.x$) $K^+$ currents in cerebellar granule neurons.[47] The drug was efficacious when applied extracellularly or intracellulary (via cell-dialysing pipette solutions). The two effects were additive, suggesting that they involved different mechanisms. The mechanisms underlying the observed current increase remain unclear, since the drug had no effect on the steady-state voltage-dependent activation, while it shifted the steady-state inactivation curve to more negative potentials. The latter effect is expected to cause a decrease rather than the observed increase in peak current amplitude. Diclofenac also caused a slight acceleration of the recovery from inactivation, but this effect was unable to account for the observed increase in current amplitude, which was obtained with interpulse intervals (10 s) many orders of magnitude longer than the time constants of restitution (<10 ms). Either a prolongation of the channel open time or an increase in single channel conductance could account for the stimulatory effect on the transient outward current. The former was suggested by the apparent slowing of the current decay observed from the original current tracings. However, no quantitative analysis of the time constants of current decay was provided. It was unlikely that the stimulatory effect involved an increase in the total number of channels given the very fast time course of onset and offset of the effect upon drug application and washout, respectively.

The transient outward current is proposed to have a role in modulating neuronal excitability, but the diclofenac concentrations needed to modulate this current ($\geq 10^{-5}$ M) are likely too high to be therapeutically relevant.[47]

### *$K_V7$ currents*

Native muscarinic receptor-inhibited (M-type) $K^+$ currents in neurons are known to modulate neuronal excitability and are carried by $K_V7.2/3$ or KCNQ2/3 channels.[80] Diclofenac and meclofenamic acid have been shown to cause a voltage-dependent increase in the amplitude of whole-cell currents carried by $K_V7.2/3$ channels heterologously expressed in Chinese hamster ovary (CHO) cells.[46] Diclofenac opens $K_V7$ channels in the mouse olfactory bulb.[81] The drug effects in CHO cells are due to a concentration-dependent shift of the steady-state voltage-dependent activation to more negative potentials, and are associated with a slowing of the rate of deactivation upon repolarization following the activating depolarization.[46] Similar effects are obtained with channels heterologously expressed in *Xenopus* oocytes and with native M current present in rat cortical neurons. As a result, hyperpolarization of the resting membrane potential is induced by diclofenac in *Xenopus* oocytes as well as in rat cortical neurons, and in addition a suppression of spontaneous action potentials is obtained in the neurons.[46] It is unclear why

diclofenac and meclofenamic acid could produce prominent effects on $K_V7.x$ channels, whereas other NSAIDs of the fenamates group, such as flufenamic, mefenamic, tolfenamic and niflumic acids, had no marked activating effects.[46] Differences could be due to various potencies or to subtle molecular structural differences. In any case, this finding again indicated the presence of non-COX-mediated mechanisms of action.

Voltage-gated $K_V7.4/5$ (KCNQ4/5) $K^+$ channels are also expressed in vascular smooth muscles, where they are involved in the control of vascular tone.[82] At lower concentrations (10 μM), diclofenac had no effect on $K_V7.5$ current in vascular smooth muscle cells.[44] In contrast, at 100 μM the drug suppressed the $K_V7.5$ current, and at the same time enhanced the $K_V7.4$ currents.[48] Mutation of residues in the voltage-gating sensor of $K_V7.5$ did not remove the blocking properties of diclofenac, hence the site of action of the drug on $K_V7.5$ channels remains unclear.[48] The $K_V7.5$ channel inhibitory effect of diclofenac is also not a common feature of NSAIDs or a reflection of their COX selectivity, since the COX-2-selective NSAID rofecoxib had no blocking effect, but celecoxib inhibited the channels even more potently than diclofenac.[44,83]

### *Ligand-gated $K^+$ channels*

A large body of indirect evidence from behavioural studies in animal models of pain supports a possible role of diclofenac as an opener of ligand-gated $K^+$ channels like ATP-sensitive $K^+$ ($K_{ATP}$) and $Ca^{2+}$-activated $K^+$ ($K_{(Ca)}$) channels.[49–54] The opening of $K_{ATP}$ channels has been proposed to mediate the peripheral (i.e. at primary afferent neuron level) or central (i.e. at spinal level) antinociceptive effects of diclofenac in rat models of $PGE_2$-mediated hyperalgesia and formalin-induced pain.[49,51–54,84] Hence, the sulfonylurea drug, glibenclamide, which blocks $K_{ATP}$ channels, also suppressed the analgesic action of diclofenac in rats injected subcutaneously with formalin.[51–53] In addition, the concurrent activation of large conductance $Ca^{2+}$-activated ($BK_{(Ca)}$) and small conductance $Ca^{2+}$-activated ($sK_{(Ca)}$) by diclofenac is implicated in antinociceptive effects.[51,84]

### *ATP-sensitive channels*

There is no direct evidence, such as can be derived from voltage clamp studies, of an effect of diclofenac on $K_{ATP}$ channels. As stated above, evidence that NSAIDs may act on these channels is only indirect and derived from functional studies measuring responses such as nociception and vasodilatation, which are affected by activators and blockers of $K_{ATP}$ channels.

$K_{ATP}$ channels affect the excitability of neuronal cells and their modulation by various physiological and pathological factors may therefore interfere with various sensory mechanisms, including nociception. $K_{ATP}$ channels can be activated by nitric oxide (NO), which can act on the channels either indirectly via a pathway involving the production of cGMP and the activation of protein kinase G, or directly via a nitrosylation of the channel proteins.[85,86] The diclofenac mode of action on the ligand-gated $K^+$ channels has been proposed to involve nitric oxide synthase (NOS) and cGMP, since the antinociceptive effects are prevented by inhibitors of NOS or of guanylate cyclase.[49–52,54] Diclofenac also has synergistic actions on peripheral antinociceptive effects of sildenafil, a known inhibitor of cGMP-specific phosphodiestrases.[87,88] Interestingly, the NOS-mediated central and peripheral antinociceptive effects of diclofenac can be additive.[52] The antinociceptive effects of ketorolac are also proposed to be mediated by the NO-cGMP pathway.[89]

The mechanism underlying the diclofenac or ketorolac effect on the NO-cGMP-PKG pathway remains unclear. In cerebral vessels, diclofenac inhibits the hypercapnia- and acid-induced upregulation of endothelial NOS (eNOS) mRNA expression.[90] Regarding other possible mechanisms of action, COX inhibition does not seem to be critical in mediating peripheral antinociceptive effects as indometacin is only effective in some pain models but not others.[49,51,54,84]

The role of opioid receptors in mediating the diclofenac antinociceptive effects is also unclear. The central antinociceptive effects of diclofenac are known to be reversed by the opioid receptor antagonist naloxone, but naloxone has no effect on diclofenac-induced peripheral antinociceptive effects.[28,49,54]

In the above-mentioned pain models, the diclofenac antinociceptive effects were attributed to $K^+$ channel opening mainly on the basis of the reversal of effects by specific channel inhibitors e.g. by glibenclamide. A potential drawback of such extrapolations is that the target-specificities of the inhibitors *in vivo* are not well-known. For example, drugs like glibenclamide may, as expected, induce hypoglycaemia, thereby creating potentially confounding factors, not necessarily linked to the $K_{ATP}$ channels in nociceptors. Furthermore, in one study involving human skin blood vessels, diclofenac (which is proposed to promote $K^+$ channel opening in nociceptors) could not oppose the effects of the $K_{ATP}$ channel inhibitor glibenclamide in decreasing acetylcholine-stimulated vasodilatation.[91] There is, therefore, still a need for direct electrophysiological characterization of the effects on ATP-sensitive $K^+$ channels.

### *$Ca^{2+}$-activated and $Na^+$-activated channels*

Just like in the case of $K_{ATP}$ channels, there is very little direct voltage clamp evidence of an effect of NSAIDs on $Ca^{2+}$-sensitive $K^+$ channels. Blockers of $Ca^{2+}$-sensitive $K^+$ channels such as charybdotoxin and apamin, when administered locally or intrathecally, abolished the antinociceptive effect of diclofenac or of the structurally related COX-2 antagonist

lumiracoxib.[92] The fenamates niflumic acid, meclofenamic acid and tolfenamic acid stimulated large-conductance $Ca^{2+}$-sensitive $K^+$ channels in osteoblast-like cells by shifting the activation curve to hyperpolarized potentials.[93] In contrast, piroxicam and NS 398 did not have similar effects.

$Na^+$-sensitive $K^+$ (Slo2.1) channels are related to $Ca^{2+}$-sensitive $K^+$ channels and are activated by an increase of intracellular $Na^+$ concentration, as well as an increase of intracellular $Cl^-$ or a decrease of intracellular ATP.[94] Niflumic acid and flufenamic acid are strong activators of the channels.[94]

### *Acid-sensing ion channels (ASICs)*

Acid-sensing is mediated by capsacin-insensitive- (ASIC) and by capsaicin-sensitive (TRPV1) channels. ASICs play an important role in sensing of pain associated with inflammation. Their number is increased during inflammation in neurons expressing them under basal conditions, and novel expression appears in neurons not initially harbouring them.[36] In addition, their activation may contribute to other types of cellular injury, for example during ischaemia.[95] Various isoforms of ASICs exist and are probably differently expressed in various cells.

NSAIDs such as salicylic acid, ibuprofen and diclofenac have been shown to inhibit ASICs in nociceptors and in cortical neuronal cells as well as in bone-marrow-derived dendritic cells.[36,57,95] The effects of salicylic acid were obtained at clinically-relevant concentrations, indicating that these effects may contribute to the analgesic and cell protection action of the NSAID. The inhibitory actions of the various drugs in nociceptors was accompanied with a suppression of excitability, and in cultures of cortical neurons it resulted in decreased lactate dehydrogenase release during cell incubation in acid medium, reflecting decreased acid-induced cell damage. Those results may help explain antinociceptive actions of NSAIDs persisting in COX-knock-out animals, and the effects of aspirin may contribute to its clinical usefulness during brain ischaemia following stroke. NSAIDs such as piroxicam, tolmetin, etodolac, nimesulide, naproxen, indometacin or paracetamol (acetaminophen) have no effect on ASIC1a or ASIC3 activity.[36] Among the other NSAIDs, flurbiprofen inhibits acid-mediated intracellular $Ca^{2+}$ increase in rat brain cells, an effect that may account for the drug's cerebral tissue protection observed during ischaemia/reperfusion.[96]

Diclofenac has a variety of effects on ASICs, including changes in the properties of ASIC currents ($I_{ASIC}$), modulation of proton sensitivity, and changes in protein expression. The drug suppresses endogenous $I_{ASIC}$ in hippocampal interneurons and in bone marrow-derived dendritic cells, as well as currents due to heterologously-expressed channels in COS or CHO cells.[36,56,57] In hippocampal interneurons, diclofenac does not compete with protons since the activating half-maximum pH is unchanged.[56] The effect of diclofenac is voltage-independent, in contrast with the effect of inhibitors such as amiloride, which displays voltage-dependence. Its blocking effect is attenuated in the absence $Ca^{2+}$, suggesting possible interactions between protons, the divalent cation and the drug.[56] In addition to decreasing the current amplitude, there is a slowing of the time course of $I_{ASIC}$ decay. Diclofenac alters the pH-sensitivity of the desensitization process, which occurs at more alkaline pH, and slows the recovery from desensitization while having no significant effect on the time course of onset of desensitization.[56] In heterologous expression systems, diclofenac suppresses currents due to ASIC1a/2a, ASIC2a, and ASIC3 channels in CHO cells, and only ASIC3 currents in COS cells, but the explanation for this difference is unclear since the same channel isoform was expressed.[36,56] One possible explanation is the difference in diclofenac concentrations tested in the two studies (lower in the study of COS cells) but the expression system itself may possibly have an influence on the concentration–response relationship. The inhibitory effects of diclofenac on ASIC3 in COS cells and ASIC in hippocampal interneurons are likely to be independent of COX inhibition, given that not all NSAIDs tested had inhibitory effects on the currents.[36,56] Apart from the actions on ASIC activity, diclofenac inhibits the inflammation-induced mRNA expression of ASIC in DRG neurons.[36] In dendritic cells, the drug prevents the acid-induced upregulation of cell surface markers that are normally associated with cell maturation.[57]

It is interesting to note the homology between the voltage-dependent inactivation of $Na_V$ channels and the desensitization of ASIC currents. Both result in the slow decline of current but are induced by different factors, transmembrane voltage or protons, respectively. For ASIC, acidification is equivalent to depolarization, and the more alkaline the pH the more ASIC channels are available. Similar to the effect on voltage-dependent channels where channel availability is decreased by diclofenac (half-maximum inactivating voltage shifted to more negative potentials), for ASIC, channel availability is also decreased (half-maximum inactivating pH shifted to higher pH values) by the drug. The slowed decay of $I_{ASIC}$ despite increased extent and unaltered kinetics of onset of desensitization could possibly be explained by a delayed and/or prolonged opening of the channels during acidification. This would account for the observed slowing of $I_{ASIC}$ decay by ibuprofen, despite it having no effect on inactivation, and for the increased time to peak current.[56]

Functionally, the inhibitory effects of diclofenac on ASICs may partly account for the decrease in $CO_2$-evoked responses seen in corneal polymodal nociceptors.[42] The drug effects may also mediate the modulation of acid-induced pain transduction observed in humans since topical application of the drug decreases such type of pain.[97] However, in airway

tissue, diclofenac has no effect on the acid-induced tracheal ring relaxation likely to be mediated by ASICs.[98]

### *Transient receptor potential and other channels*

Transient receptor potential (TRP) channels are a new group of cation channels found in several tissues and are involved in mediating sensory signalling and various other physiological and disease processes.[99] The effects of NSAIDs on TRPs and related channels are increasingly being studied.

TRPs include the ankyrin 1 subgroup (TRPA1) channels (previously known as ANKTM1), which are temperature (cold)-sensitive and are particularly expressed in nociceptor neurons.[100] Fenamate NSAIDs (such as flufenamic acid, niflumic acid), diclofenac and indometacin activate TRPA1 channels heterologously expressed in *Xenopus* oocytes and promote TRPA1-mediated $Ca^{2+}$ loading in lung fibroblasts.[58] Other NSAIDs like naproxen and ketorolac are ineffective, suggesting COX-independent mechanisms.[58] Instead, given that flufenamic acid amplified the stimulatory actions of cold temperature, allosteric actions or synergistic interactions among the TRPA1 channel activators may play a role.[58]

A recent study investigated the effect of diclofenac and ibuprofen on TRPC6 channel activity recorded using the cell-attached patch method in podocytes of freshly isolated glomeruli and showed that both drugs decreased the open probability of the channel.[101] The single channel conductance was not modified by the drugs. Gain-of-function mutations of TRPC6 are associated with focal segmental glomerulosclerosis, probably due to a dysfunction of podocytes, and NSAIDs have been reported to have beneficial action in this condition. It is therefore likely that the NSAID effect on TRPC6 could mediate this beneficial effect. However, since only one drug concentration (500 μM) was used in the above study, more work will be needed to establish the concentration–response relationship and clarify its relevance to therapeutic concentrations. It remains unclear whether the observed effect was related to COX inhibition.

Diclofenac is reported to have no effect on capsaicin-induced (i.e. TRPV1-mediated) relaxant effect on ileum smooth muscles.[102] Since neuronal TRPV1 is implicated in thermo-sensing and in acid-sensing, the modulation of these sensations by diclofenac, as in the case for acid-sensing, implies the involvement of additional receptors in the sensing function (e.g. implication of both TRPV1 and ASIC channels in acid-sensing). In mice, pretreatment with diclofenac had no effect on menthol-induced (i.e. TRPM8-mediated) hyperthermia.[103] It is doubtful whether the NSAID effect on TRPA1 represents a common feature of the drugs on temperature-sensitive TRPs given that NSAIDs containing the anthranilic acid ring inhibit rather than stimulate the thermosensitive channels TRPV1, TRPV3 and TRPM8.[58] Flufenamic acid inhibits other TRPs like TRPC3, TRPC5, TRPC7, TRPM2, and TRPM4 channels (see Clapham[104]) and activates TRPC6 channels.[105] Although diclofenac and flufenamic acid are structurally related considering that they both contain a core phenyl-amino-phenyl ring, it is not 11 uncommon for them to display different effects on the same type of channel. In a recent study, we showed that flufenamic acid and the related drug ACA were able to induce a cation nonselective current in cardiac ventricular myocytes, whereas diclofenac was ineffective.[59]

In erythrocytes, diclofenac is proposed to modulate apoptotic cell death by inhibiting a stress-activated $Ca^{2+}$-permeable channel, thereby preventing $Ca^{2+}$ overload.[60,106] The drug suppresses whole-cell cation currents induced by the removal of extracellular chloride or by $PGE_2$ and decreases intracellular $Ca^{2+}$ accumulation.[60,106] The channels appear to be linked to $Ca^{2+}$ signalling processes as the currents and the $Ca^{2+}$ loading are greater in mice deficient in annexin, a $Ca^{2+}$-binding regulator protein.[106] Diclofenac is proposed to act partly via the inhibition of COX, since $PGE_2$ is involved and the above-mentioned effects are inhibited by aspirin.[60,106] The drug effects may affect other arachidonic acid metabolites, given that inhibition of lipoxygenase enhances the $PGE_2$-induced channel activity and that these lipoxygenase-mediated effects are sensitive to diclofenac.[107]

In rodent melanoma cells, diclofenac increases the amplitude of a $Ca^{2+}$-regulated $K^+$ current. The current is inhibited instead of being activated by intracellular $Ca^{2+}$, and the underlying channels were proposed to belong to the EAG 12 family of channels and to be implicated in cell migration.[55] However, the nature of the channels remains uncertain since the observed currents could be due to other channel types that are activated under similar conditions. TRPM7 channels are sensitive to intracellular divalent cations and, although they are nonselective, they carry outward $K^+$ currents at positive potentials (see Gwanyanya *et al.*[69,108] and Kozak *et al.*[109]). The possibility that the $Ca^{2+}$-sensitive currents recorded in melanoma cells are carried by TRPM7 channels needs to be considered given the expression of TRPM7 and its role in survival in these cells.[110] The mode of action of diclofenac in the melanoma cells may involve modulation of endothelin receptors ($ET_C$), since diclofenac reverses both the endothelin 1-induced decrease in the $K^+$ current and the endothelin 1-associated decrease in cell migration.[55]

Various NSAIDs, including salicylate, ibuprofen, sulindac, and indometacin all inhibit store-operated $Ca^{2+}$ entry (SOCE) in smooth muscle (without affecting depolarization-induced $Ca^{2+}$ entry due to the opening of voltage-sensitive $Ca^{2+}$ channels). Similar effects are obtained with *R*-flurbiprofen, which has no anti-inflammatory action, suggesting a dissociation of this effect from COX inhibition. SOCE is mediated by so-called $Ca^{2+}$-release activated $Ca^{2+}$ (CRAC) channels in association with Orai. However, it has been shown that the NSAIDs do not directly inhibit the

current carried by the CRAC channels ($I_{CRAC}$) but do so by preventing mitochondria from taking up $Ca^{2+}$ (hence enhancing $Ca^{2+}$-dependent inactivation). This inhibitory action on SOCE seems to be associated with an antiproliferative action.[71]

### *Anion channels*

The NSAID ibruprofen improves symptoms in cystic fibrosis, a disease due to mutations in the cAMP-gated, $Cl^-$ selective cystic fibrosis transmembrane conductance regulator (CFTR) channels. Earlier studies indicated that ibuprofen (and also salicylate and aspirin, both at much higher concentrations) inhibited CFTR channels (by apparently reducing single channel conductance) in respiratory epithelial cells.[111] Ibuprofen inhibits CFTR-mediated $Cl^-$ secretion, a result which cannot account for the beneficial effect of this drug in cystic fibrosis but which instead would predict aggravation. Similarly, aspirin, indometacin and ibuprofen decreased the CFTR gene expression and suppressed cAMP-stimulated currents in carcinoma cells.[112] However, the effect of NSAIDs may depend on the basal stimulation of the channel. In human epithelial cells ibuprofen either suppresses or stimulates CFTR $Cl^-$ currents depending on the concentration of intracellular cAMP.[113] Under high cAMP stimulation levels, ibuprofen inhibits the channel in a voltage-dependent manner, since inhibition is higher at negative potentials. This inhibitory effect is mediated from the extracellular side of the membrane and may involve channel occlusion. In contrast, at low cAMP-mediated stimulation or under perforated patch recording conditions (which minimize perturbations of the intracellular medium) ibuprofen enhances CFTR currents. Similar effects could be obtained with meflofenamic acid. Interestingly, the stimulatory effect was obtained at clinically relevant concentrations (5 μM), whereas much higher concentrations were needed for the inhibitory action. While this stimulatory action of NSAIDs on CFTR function may be beneficial, a decrease of the expression of the channels by the same drugs may at least partly counteract the beneficial effect.[112]

Fenamate drugs such as flufenamic acid are known to inhibit various $Cl^-$ currents, and so diclofenac, which presents some similarity with these drugs, could be expected to block these currents. However, we found that diclofenac did not inhibit the $Ca^{2+}$-activated $Cl^-$-based transient outward current in cardiac ventricular myocytes, even in cells where the current could be suppressed by flufenamic acid or by ACA.[114] Similarly, diclofenac did not inhibit the GABA-induced activation of $Cl^-$ currents in sensory neurons, in contrast to other NSAIDs such as indometacin or ibuprofen-like drugs.[115] The co-administration of these latter NSAIDs enhanced the convulsive potency of drugs such as quinolone-based antibiotics, an effect related to the inhibition of the binding of GABA to its receptor. Therefore, diclofenac appears safer in combined therapy with these antibiotics.

## Therapeutic and toxicological implications

### Drug effects

The drug actions on ion channels outlined above may account for several therapeutic or adverse effects not directly linked to classic NSAID actions. On the one hand, NSAIDs may have short-term beneficial effects such as peripheral and central analgesia, regulation of cell excitability and possibly anticonvulsant activity. In addition, by limiting $Ca^{2+}$ influx and the associated cell damage, the drugs may afford cellular protection. The effect on $Ca^{2+}$ influx can also influence vascular tone. The intermediate- to long-term effects arising from transcriptional modifications and alterations in protein synthesis include modulation of apoptosis and of inflammation-induced immune responses, as well as modulation of cell proliferation and of cell migratory patterns. On the other hand, possible adverse effects include neurological dysfunction or cardiac arrhythmogenesis due to excessive inhibition of $Na^+$ and $Ca^{2+}$ channels.

For the more extensively studied NSAID diclofenac, a key question is whether the effects on ion channels are expected to occur under therapeutic conditions. Given that the IC50 13 value of diclofenac for COX-1 inhibition is 0.2 μM, whereas that for COX-2 is 0.6 μM, and given that the peak plasma concentrations of the drug under clinical conditions can be approximately 5–10 μM, most of the therapeutic effects could be attributed to COX inhibition.[116–118] In many studies, the doses of diclofenac needed *in vivo* to modulate ion channels are high, e.g. 18 mg/kg (approximately ≥90 μM) to induce antinociceptive effects in rats and an ED50 value of 43 mg/kg 14 (approximately ≥200 μM) for anticonvulsant effects in mice.[46,53] While most of the effects on ion channels seem to occur at nontherapeutic concentrations, those on some neuronal and myoblast $Na^+$ channels ($K_d$ = 7–14 μM) as well as on leucocyte $K_V1.3$ channels ($K_d$ = 2.6 μM) occur at concentrations compatible with a clinical effect (see Table 1). Higher local concentrations can be obtained following topical (e.g. skin) applications and could induce local COX-independent effects. In addition, the drug is also highly bound to proteins and undergoes hepatic first-pass metabolism, suggesting that the effective drug concentration could be overestimated when calculated on the basis of the total drug dose administered in in-vivo studies.[117] Furthermore, for synergistic effects with other drugs or for changes in gene expression, minimal concentrations may suffice. As an example, as discussed earlier, diclofenac potentiates the effect of ineffective concentrations of sildenafil in inducing antinociceptive effects.[87] Finally, for many of the channels, the effective concentrations of diclofenac have not been determined yet.



The mechanisms underlying the NSAID effects on ion channels are diverse (see Figure 2), and in most cases appear to be independent of COX inhibition.[66,67,119] One such mechanism of action involves direct drug binding to induce block, to induce allosteric effects, or to produce synergistic or antagonistic actions with other channel modulators. This appears to be the case with voltage-gated $Na^+$, voltage-gated L-type $Ca^{2+}$, some voltage-gated $K^+$, ASICs, TRPs and other nonselective cation channels. Secondly, diclofenac may indirectly affect channels by interacting with the production of second messengers like NO and cGMP as is probably the case for ligand-gated $K^+$ channels. Thirdly, the modulation of the expression of channel proteins and of regulatory molecules may play a role in $K_V$ channels, ASICs and $Ca^{2+}$-regulated $K^+$ channels. For some channels, however, there may be cross-talk between COX inhibition and the other NSAID effects. This may be the case for stress-activated $Ca^{2+}$-permeable channels in erythrocytes, where the channels are sensitive to a drug like diclofenac but can also be activated by $PGE_2$, a downstream metabolite of COX activity.[60,106] The same could apply to channels like $K_V1.3$ and ASICs.[36,45]

## Contribution of channel effects to the anticonvulsive and antalgic actions

Functionally, it has been proposed that the local anaesthetic-like, $I_{Na}$ blocking, actions of diclofenac in neurons may account for its suppression of mechanically-stimulated pain in masseter muscle nociceptors.[120] In addition, topical application of the drug decreases noxious stimuli-evoked responses in corneal polymodal nociceptors.[42]

Diclofenac shares the same effect on neuronal channels with carbamazepine and with local anaesthetics, and at the same time is able to activate $K_V7$ channels, and so the drug may be expected to have anticonvulsive action. Its affinity of binding on $Na^+$ channels is even higher than that of carbamazepine, suggesting that at doses given clinically (1–2 mg/kg) it may be more active than carbamazepine. Indeed, diclofenac has been shown to have anticonvulsant activity in mice, but so far the contribution of the $I_{Na}$ inhibition to this effect is not known, and the anticonvulsive effect has been attributed only to the activation of $K^+$ channels.[46] Diclofenac suppresses spontaneous or evoked cortical neuron activity and has anticonvulsant activity in mice, as assessed using electroshock tests, but the effects have not yet been linked directly to the activity of M-type $K^+$ channels.[46] Nonetheless, for these anticonvulsant effects of diclofenac, certain structural components of the drug seem to be important. For example, its ester derivative (named compound 15) shows anticonvulsant effects, whereas the amide derivative (named compound 6) has none.[72]

## Future directions

Considering the ill fate of COX-2-selective NSAIDs like rofecoxib, the key to the continued clinical use of NSAIDs is likely to depend on their cardiovascular safety profile. It is known that several NSAIDs are associated with a high cardiovascular risk.[19,20,32,121] As stated above, the cardiovascular morbidity of COX-2-selective drugs has tentatively been attributed to atherogenic effects (see Joshi *et al.*[23]). The expression of COX-2 is also known to be important in the cardioprotection produced by ischaemic or pharmacological preconditioning.[122,123] However, diclofenac and other nonselective NSAIDs still cause long-term cardiovascular morbidity at levels as high as those of COX-2-selective drugs.[121] Thus, the cardiovascular adverse effects of NSAIDs require further elucidation. In the liver, diclofenac induces hepatotoxicity via the opening of a channel-like structure in the mitochondrial inner membrane called the mitochondrial permeability transition pore, a pathway of entry of lethal substances into the mitochondria during oxidative stress.[124,125] Such a pore is present in cardiac cells, where its closure during ischaemia is known to promote cardioprotection via the process of ischaemic preconditioning.[126,127] Currently, the effect of diclofenac on the mitochondrial permeability transition pore in the heart is not known. As for conventional cardiac channels, we have shown here that diclofenac and fenamates inhibited cardiac $Na^+$ channels (see Yarishkin *et al.*[43]). Also, we have shown here, as well as in previous studies, that the drugs decreased the amplitude and shortened the duration of ventricular action potentials.[59] This effect is similar to that of local anaesthetics and as such may, to some extent, be anti-arrhythmic. However, pro-arrhythmic actions may result from excessive changes. Taken together with the likelihood that diclofenac and other NSAIDs could inhibit cardiac $Ca^{2+}$ channels and $K_V$ channels, this may account for increased cardiac mortality if arrhythmias and cardiac contractile dysfunction are induced.[43,75]

As mentioned above, some effects of diclofenac can be attributed to specific structural components present in other drugs (Figure 1a). So far, some derivatives of diclofenac have already been shown to exhibit differences in COX-inhibiting activity or in inducing anticonvulsant effects as well as to have substantial antimycobacterial activity.[72,128] The drug may therefore serve as a template for the development of new ion channel modulators that are in the form of nonselective NSAIDs.

## Conclusions

Several NSAIDs have therapeutic and adverse effects (including cardiovascular morbidity), some of which may be due to their actions on ion channels. Many of these effects of ion channels are unrelated to COX inhibition and include drug–channel interactions, the release of second messengers and

changes in gene expression. As more knowledge of such mechanisms of action becomes available, the NSAIDs' therapeutic or adverse effects will become better understood.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.


### Funding

This study was supported by Grant G.0634.07 from FWO, the Flemish Foundation for Science. A. Gwanyanya was supported by the Belgian Technical Cooperation and UCT/ Carnegie Research Development Grant.


## References


1. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 1971; 231: 232–235.
2. Botting RM. Vane's discovery of the mechanism of action of aspirin changed our understanding of its clinical pharmacology. *Pharmacol Rep* 2010; 62: 518–525.
3. Vane JR *et al.* The mechanism of action of aspirin. *Thromb Res* 2003; 110: 255–258.
4. Hemler M *et al.* Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *J Biol Chem* 1976; 251: 5575–5579.
5. Xie WL *et al.* Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci U S A* 1991; 88: 2692–2696.
6. Patrono C. Aspirin: new cardiovascular uses for an old drug. *Am J Med* 2001; 110: 62S–65S.
7. Giovannucci E *et al.* Aspirin and the risk of colorectal cancer in women. *N Engl J Med* 1995; 333: 609–614.
8. Gupta RA *et al.* Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer* 2001; 1: 11–21.
9. Pasinetti GM. Cyclooxygenase and inflammation in Alzheimer's disease: experimental approaches and clinical interventions. *J Neurosci Res* 1998; 54: 1–6.
10. Subbaramaiah K *et al.* Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Trends Pharmacol Sci* 2003; 24: 96–102.
11. Liu H *et al.* Anticancer effect of celecoxib via COX-2 dependent and independent mechanisms in human gastric cancers cells. *Dig Dis Sci* 2009; 54: 1418–1424.
12. Lanza FL *et al.* The effects of ibuprofen, indomethacin, aspirin, naproxen, and placebo on the gastric mucosa of normal volunteers: a gastroscopic and photographic study. *Dig Dis Sci* 1979; 24: 823–828.
13. Patrono C. The role of prostaglandin synthesis inhibition in the renal syndromes associated with non-narcotic analgesics. *Med Toxicol* 1986; 1(Suppl. 1): 23–33.
14. Warner TD *et al.* Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. *Proc Natl Acad Sci U S A* 1999; 96: 7563–7568.
15. Harirforoosh S *et al.* Renal adverse effects of nonsteroidal anti-inflammatory drugs. *Expert Opin Drug Saf* 2009; 8: 669–681.
16. Bombardier C *et al.* Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med* 2000; 343: 1520–1528, 1522 p following 1528.
17. Schnitzer TJ *et al.* Comparison of lumiracoxib with naproxen and ibuprofen in the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET), reduction in ulcer complications: randomised controlled trial. *Lancet* 2004; 364: 665–674.
18. Bresalier RS *et al.* Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med* 2005; 352: 1092–1102.
19. Hernandez-Diaz S *et al.* Non-steroidal antiinflammatory drugs and the risk of acute myocardial infarction. *Basic Clin Pharmacol Toxicol* 2006; 98: 266–274.
20. McGettigan P *et al.* Cardiovascular risk and inhibition of cyclooxygenase: a systematic review of the observational studies of selective and nonselective inhibitors of cyclooxygenase 2. *JAMA* 2006; 296: 1633–1644.
21. Bertagnolli MM *et al.* Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med* 2006; 355: 873–884.
22. Solomon SD *et al.* Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med* 2005; 352: 1071–1080.
23. Joshi GP *et al.* Cardiovascular thromboembolic adverse effects associated with cyclooxygenase-2 selective inhibitors and nonselective antiinflammatory drugs. *Anesth Analg* 2007; 105: 1793–1804.
24. Capone ML *et al.* NSAIDs and cardiovascular disease: transducing human pharmacology results into clinical read-outs in the general population. *Pharmacol Rep* 2010; 62: 530–535.
25. Mitchell JA *et al.* Cyclo-oxygenase-2: pharmacology, physiology, biochemistry and relevance to NSAID therapy. *Br J Pharmacol* 1999; 128: 1121–1132.
26. Henry D *et al.* Variability in risk of gastrointestinal complications with individual non-steroidal anti-inflammatory drugs: results of a

collaborative meta-analysis. *Br Med J* 1996; 312: 1563–1566.
27. Menassé R *et al.* Pharmacological properties of diclofenac sodium and its metabolites. *Scand J Rheumatol Suppl* 1978; 22: 5–16.
28. Bjorkman R. Central antinociceptive effects of non-steroidal anti-inflammatory drugs and paracetamol. Experimental studies in the rat. *Acta Anaesthesiol Scand Suppl* 1995; 103: 1–44.
29. Mayorek N *et al.* Diclofenac inhibits tumor growth in a murine model of pancreatic cancer by modulation of VEGF levels and arginase activity. *PLoS ONE* 2010; 5: e12715.
30. Bort R *et al.* Diclofenac toxicity to hepatocytes: a role for drug metabolism in cell toxicity. *J Pharmacol Exp Ther* 1999; 288: 65–72.
31. Hickey EJ *et al.* Diclofenac induced in vivo nephrotoxicity may involve oxidative stress-mediated massive genomic DNA fragmentation and apoptotic cell death. *Free Radic Biol Med* 2001; 31: 139–152.
32. Trelle S *et al.* Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *Br Med J* 2011; 342: c7086.
33. Gong N *et al.* The aspirin metabolite salicylate enhances neuronal excitation in rat hippocampal CA1 area through reducing GABAergic inhibition. *Neuropharmacology* 2008; 54: 454–463.
34. Lu YG *et al.* Salicylate, an aspirin metabolite, specifically inhibits the current mediated by glycine receptors containing alpha1-subunits. *Br J Pharmacol* 2009; 157: 1514–1522.
35. Peng BG *et al.* Aspirin selectively augmented N-methyl-D-aspartate types of glutamate responses in cultured spiral ganglion neurons of mice. *Neurosci Lett* 2003; 343: 21–24.
36. Voilley N *et al.* Nonsteroid anti-inflammatory drugs inhibit both the activity and the inflammation-induced expression of acid-sensing ion channels in nociceptors. *J Neurosci* 2001; 21: 8026–8033.
37. Cheng L *et al.* Niflumic acid alters gating of HCN2 pacemaker channels by interaction with the outer region of S4 voltage sensing domains. *Mol Pharmacol* 2009; 75: 1210–1221.
38. Zifarelli G *et al.* Identification of sites responsible for the potentiating effect of niflumic acid on ClC-Ka kidney chloride channels. *Br J Pharmacol* 2010; 160: 1652–1661.
39. Lee HM *et al.* Diclofenac inhibition of sodium currents in rat dorsal root ganglion neurons. *Brain Res* 2003; 992: 120–127.
40. Fei XW *et al.* The non-steroidal anti-inflammatory drug, diclofenac, inhibits $Na^+$ current in rat myoblasts. *Biochem Biophys Res Commun* 2006; 346: 1275–1283.
41. Yang YC *et al.* An inactivation stabilizer of the $Na^+$ channel acts as an opportunistic pore blocker modulated by external $Na^+$. *J Gen Physiol* 2005; 125: 465–481.
42. Acosta MC *et al.* Comparative effects of the nonsteroidal anti-inflammatory drug nepafenac on corneal sensory nerve fibers responding to chemical irritation. *Invest Ophthalmol Vis Sci* 2007; 48: 182–188.
43. Yarishkin OV *et al.* Diclofenac, a non-steroidal anti-inflammatory drug, inhibits L-type Ca channels in neonatal rat ventricular cardiomyocytes. *Korean J Physiol Pharmacol* 2009; 13: 437–442.
44. Brueggemann LI *et al.* Differential effects of selective cyclooxygenase-2 inhibitors on vascular smooth muscle ion channels may account for differences in cardiovascular risk profiles. *Mol Pharmacol* 2009; 76: 1053–1061.
45. Villalonga N *et al.* Immunomodulatory effects of diclofenac in leukocytes through the targeting of Kv1.3 voltage-dependent potassium channels. *Biochem Pharmacol* 2010; 80: 858–866.
46. Peretz A *et al.* Meclofenamic acid and diclofenac, novel templates of KCNQ2/Q3 potassium channel openers, depress cortical neuron activity and exhibit anticonvulsant properties. *Mol Pharmacol* 2005; 67: 1053–1066.
47. Liu LY *et al.* Diclofenac, a nonsteroidal anti-inflammatory drug, activates the transient outward $K^+$ current in rat cerebellar granule cells. *Neuropharmacology* 2005; 48: 918–926.
48. Brueggemann LI *et al.* Diclofenac distinguishes among homomeric and heteromeric potassium channels composed of KCNQ4 and KCNQ5 subunits. *Mol Pharmacol* 2011; 79: 10–23.
49. Alves DP *et al.* Diclofenac-induced peripheral antinociception is associated with ATP-sensitive $K^+$ channels activation. *Life Sci* 2004; 74: 2577–2591.
50. Ortiz MI *et al.* Role of the nitric oxide-cyclic GMP-$K^+$ channel pathway in the antinociception induced by atrial natriuretic peptide (ANP) and diclofenac. *Proc West Pharmacol Soc* 2002; 45: 174–177.
51. Ortiz MI *et al.* The NO-cGMP-$K^+$ channel pathway participates in the antinociceptive effect of diclofenac, but not of indomethacin. *Pharmacol Biochem Behav* 2003; 76: 187–195.
52. Ortiz MI *et al.* Additive interaction between peripheral and central mechanisms involved in the antinociceptive effect of diclofenac in the formalin test in rats. *Pharmacol Biochem Behav* 2008; 91: 32–37.
53. Leon-Reyes MR *et al.* Pharmacokinetic of diclofenac in the presence and absence of glibenclamide in the rat. *J Pharm Pharm Sci* 2009; 12: 280–287.
54. Tonussi CR *et al.* Mechanism of diclofenac analgesia: direct blockade of inflammatory sensitization. *Eur J Pharmacol* 1994; 251: 173–179.
55. Liu LY *et al.* ET-1 inhibits B-16 murine melanoma cell migration by decreasing $K^+$ currents. *Cell Motil Cytoskeleton* 2004; 58: 127–136.
56. Dorofeeva NA *et al.* Mechanisms of non-steroid anti-inflammatory drugs action on ASICs expressed in hippocampal interneurons. *J Neurochem* 2008; 106: 429–441.
57. Tong J *et al.* Acid-sensing ion channels contribute to the effect of acidosis on the function of dendritic cells. *J Immunol* 2011; 186: 3686–3692.
58. Hu H *et al.* Activation of TRPA1 channels by fenamate nonsteroidal

anti-inflammatory drugs. *Pflugers Arch* 2010; 459: 579–592.
59. Macianskiene R *et al.* Induction of a novel cation current in cardiac ventricular myocytes by flufenamic acid and related drugs. *Br J Pharmacol* 2010; 161: 416–429.
60. Lang PA *et al.* $PGE_2$ in the regulation of programmed erythrocyte death. *Cell Death Differ* 2005; 12: 415–428.
61. Fang YJ *et al.* Arachidonic acid modulates $Na^+$ currents by non-metabolic and metabolic pathways in rat cerebellar granule cells. *Biochem J* 2011; 438: 203–215.
62. Liu Y *et al.* Inhibition of voltage-gated channel currents in rat auditory cortex neurons by salicylate. *Neuropharmacology* 2007; 53: 870–880.
63. Gould HJ, 3rd *et al.* Ibuprofen blocks changes in Na v 1.7 and 1.8 sodium channels associated with complete Freund's adjuvant-induced inflammation in rat. *J Pain* 2004; 5: 270–280.
64. Ma K *et al.* TTX-R $Na^+$ current-reduction by celecoxib correlates with changes in $PGE_2$ and CGRP within rat DRG neurons during acute incisional pain. *Brain Res* 2008; 1209: 57–64.
65. Kuo CC *et al.* Inhibition of $Na^+$ current by diphenhydramine and other diphenyl compounds: molecular determinants of selective binding to the inactivated channels. *Mol Pharmacol* 2000; 57: 135–143.
66. McCormack K *et al.* Dissociation between the antinociceptive and anti-inflammatory effects of the non-steroidal anti-inflammatory drugs. A survey of their analgesic efficacy. *Drugs* 1991; 41: 533–547.
67. Ballou LR *et al.* Nociception in cyclooxygenase isozyme-deficient mice. *Proc Natl Acad Sci U S A* 2000; 97: 10272–10276.
68. Butterworth JFT *et al.* Molecular mechanisms of local anesthesia: a review. *Anesthesiology* 1990; 72: 711–734.
69. Gwanyanya A *et al.* Magnesium-inhibited, TRPM6/7-like channel in cardiac myocytes: permeation of divalent cations and pH-mediated regulation. *J Physiol* 2004; 559: 761–776.
70. Liu Y *et al.* Salicylate blocks L-type calcium channels in rat inferior colliculus neurons. *Hear Res* 2005; 205: 271–276.
71. Munoz E *et al.* Nonsteroidal anti-inflammatory drugs inhibit vascular smooth muscle cell proliferation by enabling the $Ca^{2+}$-dependent inactivation of calcium release-activated calcium/orai channels normally prevented by mitochondria. *J Biol Chem* 2011; 286: 16186–16196.
72. Peretz A *et al.* A tale of switched functions: from cyclooxygenase inhibition to M-channel modulation in new diphenylamine derivatives. *PLoS ONE* 2007; 2: e1332.
73. Freeman LC *et al.* Depolarization and decreased surface expression of $K^+$ channels contribute to NSAID-inhibition of intestinal restitution. *Biochem Pharmacol* 2007; 74: 74–85.
74. Macias A *et al.* Celecoxib blocks cardiac Kv1.5, Kv4.3 and Kv7.1 (KCNQ1) channels: effects on cardiac action potentials. *J Mol Cell Cardiol* 2010; 49: 984–992.
75. Frolov RV *et al.* Inhibition of delayed rectifier potassium channels and induction of arrhythmia: a novel effect of celecoxib and the mechanism underlying it. *J Biol Chem* 2008; 283: 1518–1524.
76. Perianin A *et al.* Diclofenac sodium, a negative chemokinetic factor for neutrophil locomotion. *Biochem Pharmacol* 1985; 34: 3433–3438.
77. Martinez LL *et al.* Influence of verapamil and diclofenac on leukocyte migration in rats. *Hypertension* 1999; 34: 997–1001.
78. Martinez LL *et al.* Losartan attenuates the antimigratory effect of diclofenac in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 2005; 46: 190–199.
79. Amuzescu B *et al.* Cardiac ion channels and transporters. In: Georgescu A, Antohe F, eds. *From Vascular Cell Biology to Cardiovascular Medicine*. Kerala: Transworld Research Network, 2011: ••–••.
80. Brown DA *et al.* Muscarinic suppression of a novel voltage-sensitive $K^+$ current in a vertebrate neurone. *Nature* 1980; 283: 673–676.
81. Takahashi Y *et al.* Muscarinic receptor type 1 (M1) stimulation, probably through KCNQ/Kv7 channel closure, increases spontaneous GABA release at the dendrodendritic synapse in the mouse accessory olfactory bulb. *Brain Res* 2010; 1339: 26–40.
82. Yeung SY *et al.* Molecular expression and pharmacological identification of a role for $K_V7$ channels in murine vascular reactivity. *Br J Pharmacol* 2007; 151: 758–770.
83. Brueggemann LI *et al.* Novel actions of nonsteroidal anti-inflammatory drugs on vascular ion channels: accounting for cardiovascular side effects and identifying new therapeutic applications. *Mol Cell Pharmacol* 2010; 2: 15–19.
84. Ortiz MI *et al.* Pharmacological evidence for the activation of $K^+$ channels by diclofenac. *Eur J Pharmacol* 2002; 438: 85–91.
85. Chai Y *et al.* Dual regulation of the ATP-sensitive potassium channel by activation of cGMP-dependent protein kinase. *Pflugers Arch* 2008; 456: 897–915.
86. Kawano T *et al.* Nitric oxide activates ATP-sensitive potassium channels in mammalian sensory neurons: action by direct S-nitrosylation. *Mol Pain* 2009; 5: 12.
87. Asomoza-Espinosa R *et al.* Sildenafil increases diclofenac antinociception in the formalin test. *Eur J Pharmacol* 2001; 418: 195–200.
88. Boolell M *et al.* Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res* 1996; 8: 47–52.
89. Lazaro-Ibanez GG *et al.* Participation of the nitric oxide-cyclic GMP-ATP-sensitive $K^+$ channel pathway in the antinociceptive action of ketorolac. *Eur J Pharmacol* 2001; 426: 39–44.
90. Najarian T *et al.* Prolonged hypercapnia-evoked cerebral hyperemia via $K^+$ channel- and prostaglandin $E_2$-dependent endothelial nitric



oxide synthase induction. *Circ Res* 2000; 87: 1149–1156.
91. Hojs N *et al.* The effect of glibenclamide on acetylcholine and sodium nitroprusside induced vasodilatation in human cutaneous microcirculation. *Clin Physiol Funct Imaging* 2009; 29: 38–44.
92. Lozano-Cuenca J *et al.* Peripheral and spinal mechanisms of antinociceptive action of lumiracoxib. *Eur J Pharmacol* 2005; 513: 81–91.
93. Wu SN *et al.* Fenamates stimulate $BK_{Ca}$ channel osteoblast-like MG-63 cells activity in the human. *J Investig Med* 2001; 49: 522–533.
94. Dai L *et al.* Activation of Slo2.1 channels by niflumic acid. *J Gen Physiol* 2010; 135: 275–295.
95. Wang W *et al.* High doses of salicylate and aspirin are inhibitory on acid-sensing ion channels and protective against acidosis-induced neuronal injury in the rat cortical neuron. *J Neurosci Res* 2012; 90: 267–277.
96. Mishra V *et al.* Neuroprotective effect of flurbiprofen in focal cerebral ischemia: the possible role of ASIC1a. *Neuropharmacology* 2010; 59: 582–588.
97. Jones NG *et al.* Acid-induced pain and its modulation in humans. *J Neurosci* 2004; 24: 10974–10979.
98. Faisy C *et al.* Acid-induced modulation of airway basal tone and contractility: role of acid-sensing ion channels (ASICs) and TRPV1 receptor. *Life Sci* 2007; 81: 1094–1102.
99. Nilius B *et al.* Transient receptor potential cation channels in disease. *Physiol Rev* 2007; 87: 165–217.
100. Story GM *et al.* ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 2003; 112: 819–829.
101. Ilatovskaya DV *et al. Nsaids Acutely Inhibit TRPC Channels in Freshly Isolated Rat Glomeruli Biochem Biophys Res Commun.* ••, US: Elsevier Inc, 2011: 242–247.
102. Fujimoto S *et al.* Capsaicin-induced, capsazepine-insensitive relaxation of the guinea-pig ileum. *Eur J Pharmacol* 2006; 530: 144–151.
103. Tajino K *et al.* Application of menthol to the skin of whole trunk in mice induces autonomic and behavioral heat-gain responses. *Am J Physiol Regul Integr Comp Physiol* 2007; 293: R2128–R2135.
104. Clapham DE. SnapShot: mammalian TRP channels. *Cell* 2007; 129: 220.
105. Jung S *et al.* TRPC6 is a candidate channel involved in receptor-stimulated cation currents in A7r5 smooth muscle cells. *Am J Physiol Cell Physiol* 2002; 282: C347–C359.
106. Lang E *et al.* Enhanced eryptosis of erythrocytes from gene-targeted mice lacking annexin A7. *Pflugers Arch* 2010; 460: 667–676.
107. Shumilina E *et al.* Stimulation of suicidal erythrocyte death by lipoxygenase inhibitor Bay-Y5884. *Cell Physiol Biochem* 2006; 18: 233–242.
108. Gwanyanya A *et al.* ATP and $PIP_2$ dependence of the magnesium-inhibited, TRPM7-like cation channel in cardiac myocytes. *Am J Physiol Cell Physiol* 2006; 291: C627–C635.
109. Kozak JA *et al.* MIC channels are inhibited by internal divalent cations but not ATP. *Biophys J* 2003; 84: 922–927.
110. McNeill MS *et al.* Cell death of melanophores in zebrafish trpm7 mutant embryos depends on melanin synthesis. *J Invest Dermatol* 2007; 127: 2020–2030.
111. Devor DC *et al.* Ibuprofen inhibits cystic fibrosis transmembrane conductance regulator-mediated $Cl^-$ secretion. *J Clin Invest* 1998; 102: 679–687.
112. Tondelier D *et al.* Aspirin and some other nonsteroidal anti-inflammatory drugs inhibit cystic fibrosis transmembrane conductance regulator protein gene expression in T-84 cells. *Mediators Inflamm* 1999; 8: 219–227.
113. Li J *et al.* Nonsteroidal anti-inflammatory drugs upregulate function of wild-type and mutant CFTR. *Eur Respir J* 2008; 32: 334–343.
114. Gwanyanya A *et al.* Inhibition of the calcium-activated chloride current in cardiac ventricular myocytes by N-(p-amylcinnamoyl)anthranilic acid (ACA). *Biochem Biophys Res Commun* 2010; 402: 531–536.
115. Yakushiji T *et al.* Non-competitive inhibition of GABAA responses by a new class of quinolones and non-steroidal anti-inflammatories in dissociated frog sensory neurones. *Br J Pharmacol* 1992; 105: 13–18.
116. Jett MF *et al.* Characterization of the analgesic and anti-inflammatory activities of ketorolac and its enantiomers in the rat. *J Pharmacol Exp Ther* 1999; 288: 1288–1297.
117. Willis JV *et al.* The pharmacokinetics of diclofenac sodium following intravenous and oral administration. *Eur J Clin Pharmacol* 1979; 16: 405–410.
118. Leucuta A *et al.* No effect of short term ranitidine intake on diclofenac pharmacokinetics. *Rom J Gastroenterol* 2004; 13: 306–308.
119. Hunskaar S *et al.* Dissociation between antinociceptive and anti-inflammatory effects of acetylsalicylic acid and indomethacin in the formalin test. *Pain* 1986; 25: 125–132.
120. Cairns BE *et al.* Diclofenac exerts local anesthetic-like actions on rat masseter muscle afferent fibers. *Brain Res* 2008; 1194: 56–64.
121. Kearney PM *et al.* Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. *Br Med J* 2006; 332: 1302–1308.
122. Shinmura K *et al.* Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci U S A* 2000; 97: 10197–10202.
123. Alcindor D *et al.* Cyclooxygenase-2 mediates ischemic, anesthetic, and pharmacologic preconditioning in vivo. *Anesthesiology* 2004; 100: 547–554.
124. Masubuchi Y *et al.* Role of mitochondrial permeability transition in diclofenac-induced hepatocyte injury in rats. *Hepatology* 2002; 35: 544–551.
125. Gomez-Lechon MJ *et al.* Diclofenac induces apoptosis in hepatocytes. *Toxicol In Vitro* 2003; 17: 675–680.



126. Crompton M *et al*. Inhibition by cyclosporin A of a $Ca^{2+}$-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. *Biochem J* 1988; 255: 357–360.

127. Hausenloy DJ *et al*. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc Res* 2002; 55: 534–543.

128. Sriram D *et al*. Synthesis, in vitro and in vivo antimycobacterial activities of diclofenac acid hydrazones and amides. *Bioorg Med Chem* 2006; 14: 3113–3118.



| Toppan Best-set Premedia Limited | |
|---|---|
| Journal Code: JPHP | Proofreader: Mony |
| Article No: 1479 | Delivery date: 10 February 2012 |
| Page Extent: 17 | |


# AUTHOR QUERY FORM

Dear Author,
During the preparation of your manuscript, the questions listed below have arisen. Please answer **all** the queries (marking any other corrections on the proof enclosed) and return this form with your proofs.

| Query References | Query | Remark |
|---|---|---|
| 1 | AUTHOR: The new British approved name for indomethacin is indometacin, thus the latter is used in the Review as per *Journal* style. | |
| 2 | AUTHOR: Is melusid correct? | |
| 3 | AUTHOR: Please define $GABA_A$ and NMDA. | |
| 4 | AUTHOR: Please define HCN. | |
| 5 | AUTHOR: The reference citations have been renumbered from Reference 39 (originally Reference 40 in Table 1) onward, and the reference list has been renumbered accordingly. Please confirm that it is OK. | |
| 6 | AUTHOR: Please define. | |
| 7 | AUTHOR: Please define $K_d$. | |
| 8 | AUTHOR: Correct reference? | |
| 9 | AUTHOR: CHO expanded to Chinese hamster ovary cells. Correct? | |
| 10 | AUTHOR: Please define COS cells | |
| 11 | AUTHOR: Correct? | |
| 12 | AUTHOR: Please define EAG. | |
| 13 | AUTHOR: Please define IC50. | |
| 14 | AUTHOR: Please define ED50. | |
| 15 | AUTHOR: Please supply page range for Reference 79 (originally Reference 68). | |
| 16 | AUTHOR: If this is not a one-page article please supply the first and last pages for Reference 86 (originally Reference 82). | |
| 17 | AUTHOR: Please supply the city location of publisher and state instead of country for Reference 101. | |
| 18 | AUTHOR: If this is not a one-page article please supply the first and last pages for Reference 104. | |

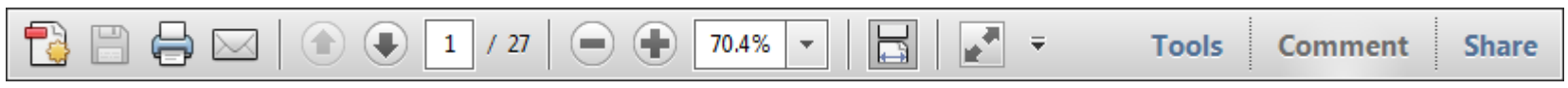
| | | |
|---|---|---|
| 19 | AUTHOR: Please can you position the superscript reference numbers next to their most relevant bit of information in the table. | |
| 20 | AUTHOR: CHO expanded to Chinese hamster ovary cells. Correct? | |

WILEY-BLACKWELL

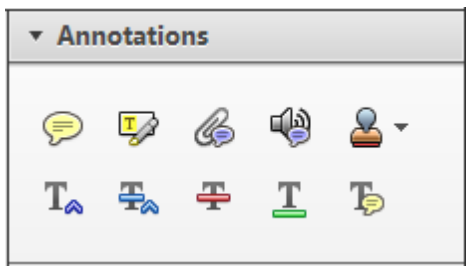
# USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

**Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)**
**The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/**

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:




This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

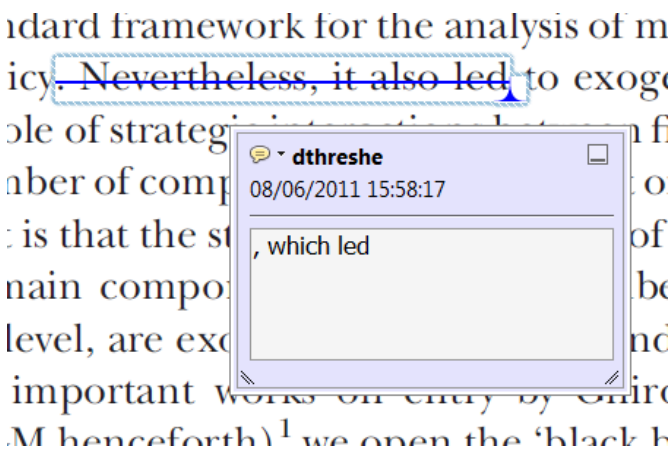



## 1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

**How to use it**

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

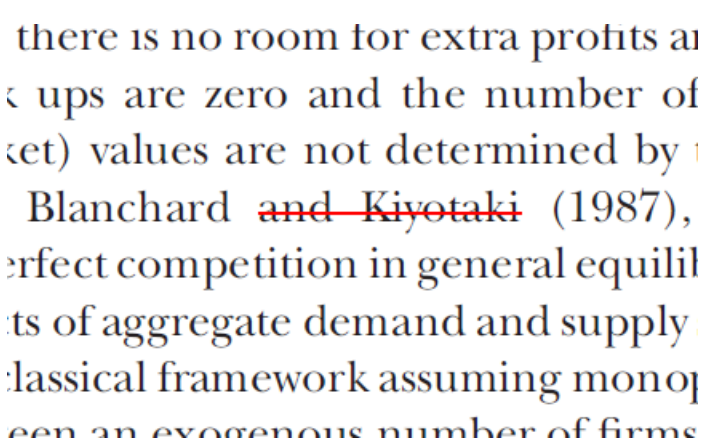



## 2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

**How to use it**

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

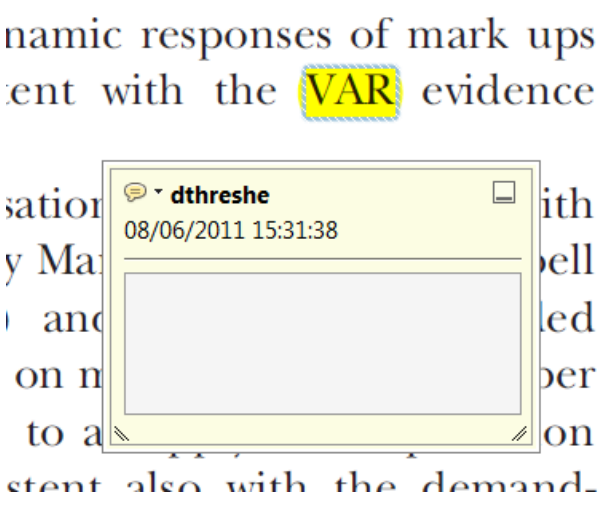


## 3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

**How to use it**

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

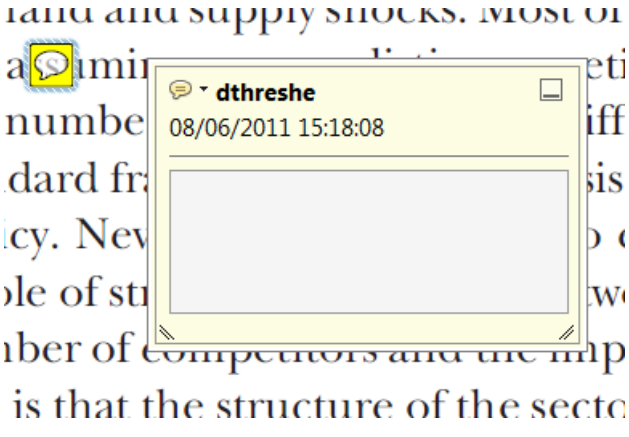



## 4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

**How to use it**

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



WILEY-BLACKWELL

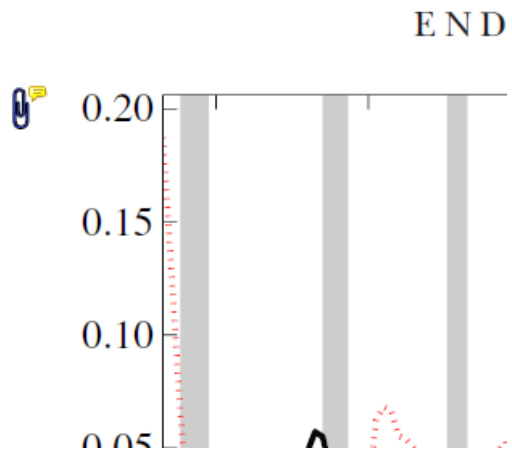
# USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

### 5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

**How to use it**

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

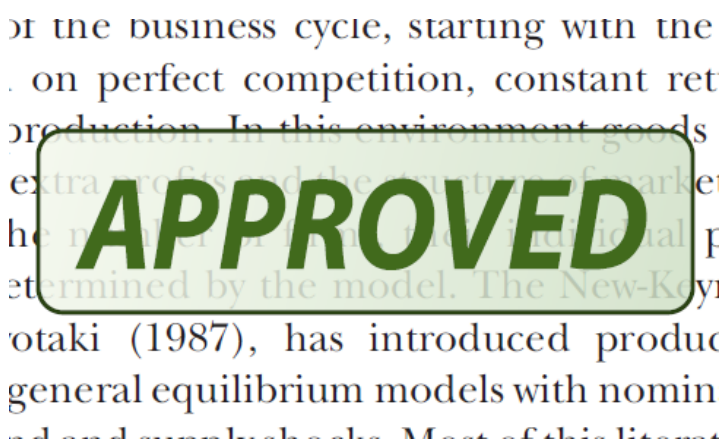


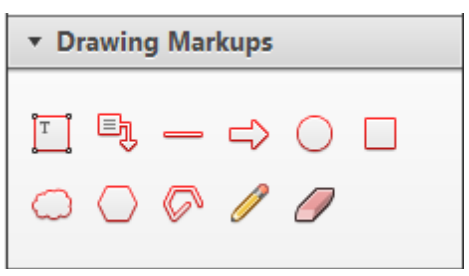

### 6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

**How to use it**

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



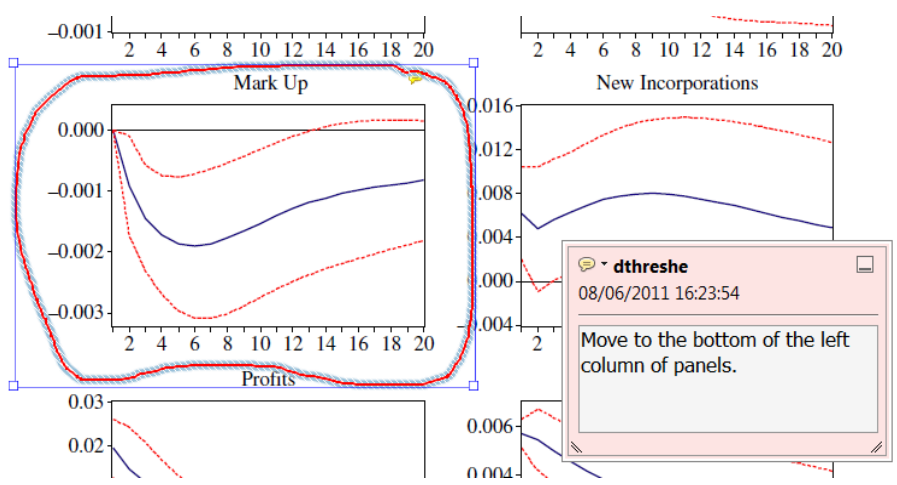




### 7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

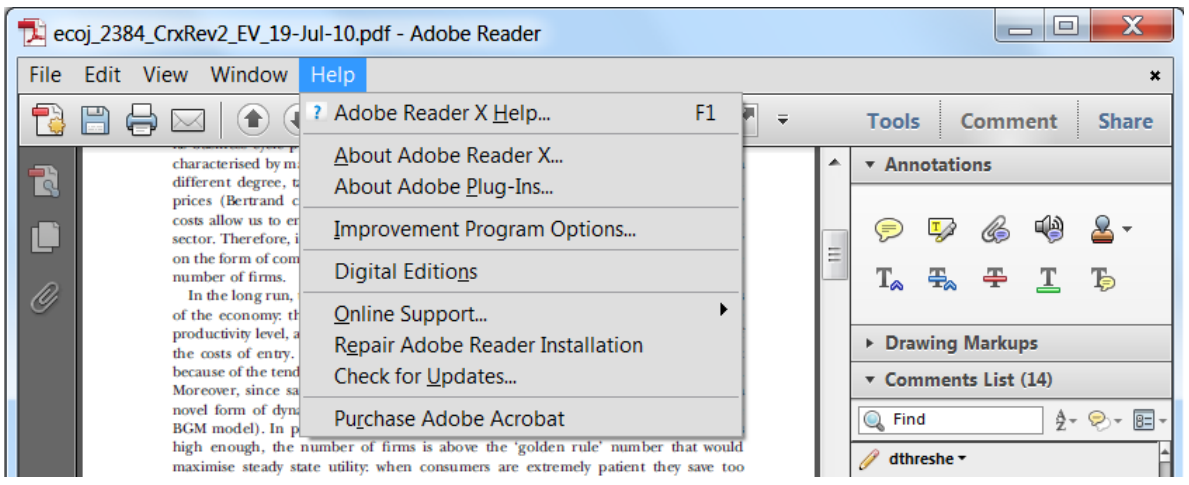
Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

**How to use it**

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.




**For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:**



# MARKED PROOF

# Please correct and return this set

Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

| *Instruction to printer* | *Textual mark* | *Marginal mark* |
|---|---|---|
| Leave unchanged | ··· under matter to remain | New matter followed by or or |
| Insert in text the matter indicated in the margin | | |
| Delete | through single character, rule or underline<br>or<br>through all characters to be deleted | or |
| Substitute character or substitute part of one or more word(s) | through letter or<br>through characters | new character or<br>new characters |
| Change to italics | under matter to be changed | |
| Change to capitals | under matter to be changed | |
| Change to small capitals | under matter to be changed | |
| Change to bold type | under matter to be changed | |
| Change to bold italic | under matter to be changed | |
| Change to lower case | Encircle matter to be changed | |
| Change italic to upright type | (As above) | |
| Change bold to non-bold type | (As above) | |
| Insert 'superior' character | through character or<br>where required | or<br>under character<br>e.g. or |
| Insert 'inferior' character | (As above) | over character<br>e.g. |
| Insert full stop | (As above) | |
| Insert comma | (As above) | |
| Insert single quotation marks | (As above) | or and/or<br>or |
| Insert double quotation marks | (As above) | or and/or<br>or |
| Insert hyphen | (As above) | |
| Start new paragraph | | |
| No new paragraph | | |
| Transpose | | |
| Close up | linking characters | |
| Insert or substitute space between characters or words | through character or<br>where required | |
| Reduce space between characters or words | between characters or words affected | |